THE EFFECT OF DENATURATING AGENTS ON THE EPR AND OPTICAL ABSORPTION SPECTRA OF PLASTOCYANINS

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1. Introduction

Plastocyanins are copper containing proteins functioning as electron carriers in the chloroplasts of photosynthetic organisms [1–5]. Their functional oxidoreductions apparently should invoive not only their copper but also in some way its ligand environment. Thus knowledge of the copper environment could be useful for the understanding of the actual mechanism of the electron transfer in the chloroplasts. Since it was clear that the copper environment was directly dependent on the protein tertiary structure, then unfolding of the protein by for example denaturation would of course affect the spatial arrangement around the metal.

The purpose of this work was to study the environmental changes in chemically denaturated plastocyanins by EPR and optical methods.

2. Materials and methods

Plastocyanins were isolated from pea leaves, wheat leaves and cucumber peelings as described earlier [6–8]. The protein preparations were electrophoretically homogeneous and had optical purity indices A_{278}/A_{597} of 1.2–1.6. Stock preparations were thawed, supplemented with ferricyanide, quickly filtered through Sephadex G-25 to remove excess oxidant and subjected to optical measurements. EPR-spectra were recorded on Varian E-4 radiospectrometer at 113°K. Solutions of $A_{597} = 1.0–1.5$ were used. Quantitation was made by double integration technique with Cu(II)EDTA as reference standard. Optical spectra were obtained on an "SF-16" instrument at room temp. Urea was recrystallized from acidified solution. Acid and alkaline denaturation was effected by 50% acetic acid or 0.5 M KOH. The solutions remained clear in both cases. Urea denaturation was carried out under anaerobic conditions. The organic solvents were chosen so as to give clear solutions with buffered water solutions of protein, and were predistilled before addition. These were dimethyl-formamide (DMFA), dimethyl sulfoxide (DMSO), pyridine, formamide, monoethanolamine and diethylamine. The protein was incubated for several hours at room temp. with 30% organic solvent.

3. Results and discussion

The EPR-spectra of native plastocyanins are given in fig. 1 and a typical optical absorption spectrum in fig. 4(1). The three proteins described here are similar to *Chenopodium album* plastocyanin [9] in having axial symmetry of the cupric site as seen from the EPR signal shape. There is only one set of hyperfine lines, and the splitting constant $A_{\parallel}$ is 60 G. This is considerably lower than $A_{\parallel}$ of the so-called Type I copper from the blue oxidases [10, 11]. The shape and inten-

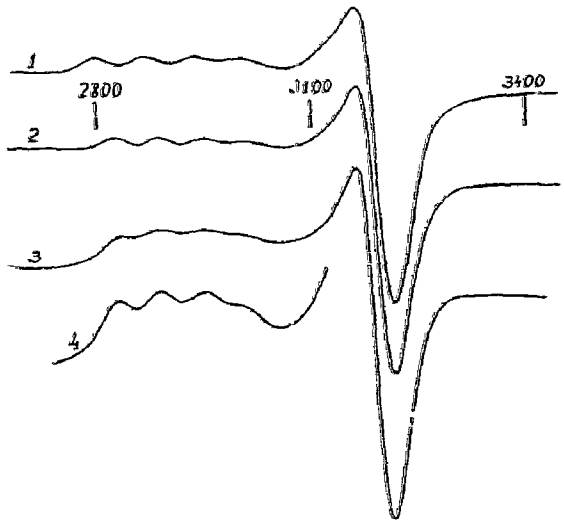

Fig. 1. EPR-spectra for 1, pea plastocyanin (A_{597} = 1.0); 2, wheat plastocyanin (A_{597} = 1.0); 3, cucumber plastocyanin (A_{597} = 1.3). 4, The low field region of the spectra of cucumber plastocyanin was recorded at an increased gain. Modulation amplitude, 6.3 G; microwave frequency, 9.12 GHz; microwave power, 10 mW.

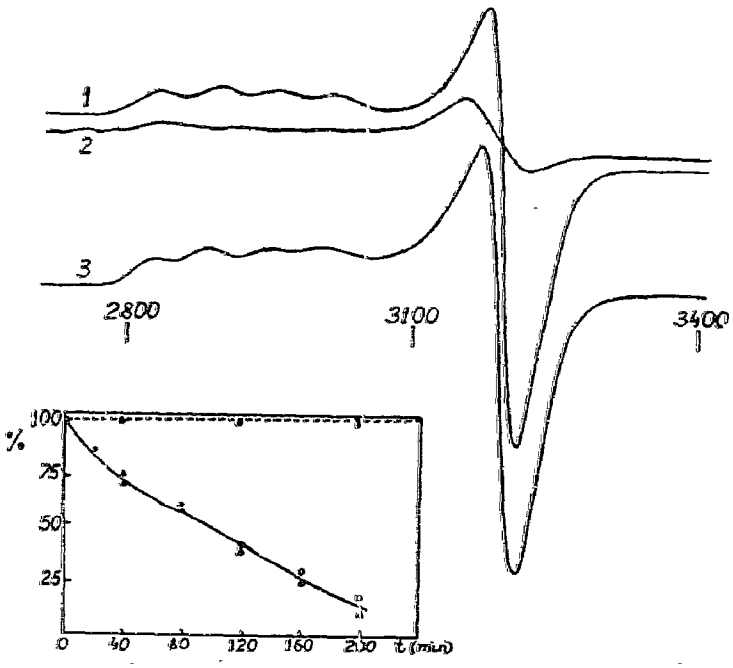

Fig. 2. The effect of 4 M urea on EPR- and optical absorption intensities for pea plastocyanin (A_{597}^{init} = 1.0). Top: 1, Immediately after addition of urea; 2, 200 min incubation with urea; 3, immediately after addition of ferricyanide to 2. Bottom: △, EPR integral intensity decrease; ○, A_{597}-absorption; ▲, EPR signal intensity immediately after ferricyanide addition to a bleached sample.

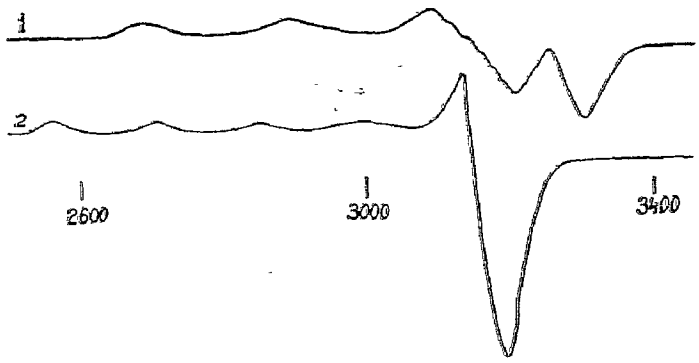

Fig. 3. EPR spectra of 1, alkaline and 2, acid denatured wheat plastocyanin. Other plastocyanins give similar spectra.

sity of EPR signals do not change in the pH range of 5.0—9.0 and at 50-fold increase of the buffer solution ionic strength. Also no essential changes in the EPR- and absorption spectra were observed on 1—2 hr incubation with 1% ionic or non-ionic detergents. However agents damaging hydrogen bonds (urea, thiourea, guanidine hydrochloride) decreased intensities of both EPR-signals and A_{597} absorbance in actually parallel fashion. Ferricyanide addition to urea bleached preparations induced an immediate rise of EPR-signal and A_{597} absorbance to initial level; the shape of the EPR-signal was also restored (fig. 2). Longer treatment with urea however makes the changes irreversible, so that after 12—16 hr incubation ferricyanide no longer can resurrect the magnetic properties of the sample or its blue colour.

Acid and alkaline denaturation quite markedly change the EPR-signal shape without changing its integral intensity. As shown in fig. 3 acid denatured plastocyanin copper retains its axial environment, but its hyperfine splitting constant $A_{\parallel}$ is now 140 κ against 60 κ of native protein. Alkali denatured protein gives an EPR-signal indicative of bi..et complexes with well resolved nitrogen superhyperfine lines [12]. Both acid and alkaline denaturation result in sample bleaching. Titration of the acid denatured protein with alkali, as well as the other case (alkali denatured protein titration with acid) does not restore the initial EPR-signal shape or A_{597} absorption.

No changes in the EPR-and visible absorption spectra of plastocyanins were observed after treatment with aprotic DMFA, DMSO or formami.., thus indicating that these solvents had not caused deep denaturation of the protein and had not come into co-ordination to plastocyanin copper.

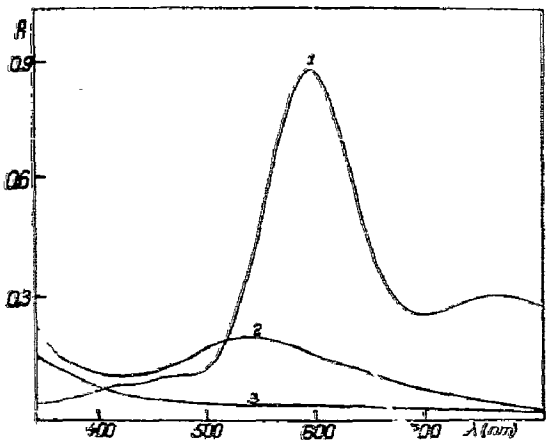

Fig. 4. Optical absorption spectra for cucumber plastocyanin. 1, Native preparation; 2, on treatment with diethylamine; 3, on the treatment with monoethanolamine.

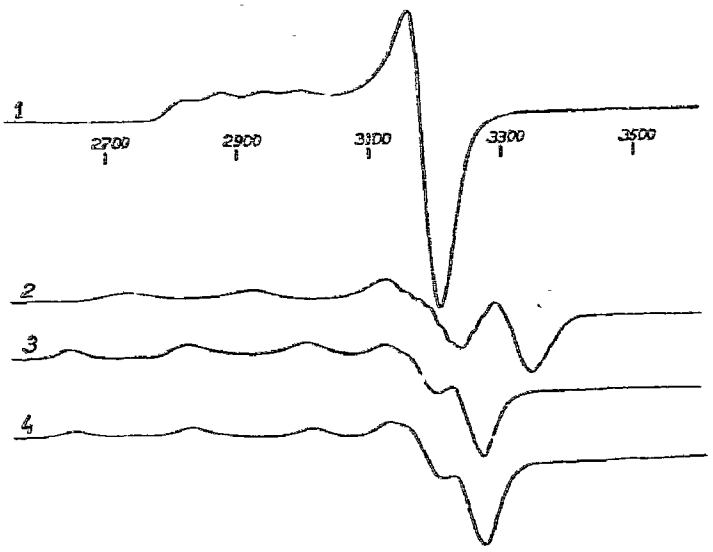

Fig. 5. EPR spectra of wheat plastocyanin treated by organic solvents. 1, DMFA; 2, diethylamine; 3, pyridine; 4, monoethanolamine.

Incubation with pyridine, monoethanolamine, diethylamine on the other hand results in a decrease of A_{597} absorption and changes in the EPR-signal shape (figs. 4, 5). Diethylamine treated plastocyanin gave EPR- and optical spectra typical for biuret complexes; results obtained with pyridine and monoethanolamine were somewhat specific. These changes in magnetic and optical properties were irreversible by dialysis or neutralization. EPR-intensity decrease observed for plastocyanins and some other electron transferring proteins containing copper on the action of denaturating agents [13, 14–17] can be visualized as resulting from cupric copper reduction by intramolecular electron donors. For plastocyanin this could be the –SH group. Perhaps the reversible reduction of the protein copper in the urea treated plastocyanin could be used as a model for the study of conformational changes involved in the electron transfer.

The proton relaxation rates [9] showing that there is no water in the coordination sphere of plastocyanin copper together with the data on organic solvents (DMSO, DMFA, formamide) we present here seem to imply that the alternate mechanism of direct electron transfer not involving internal electron donating groups probably cannot operate.

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